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PII: S0264-410X(97)00226-0

Vaccine, Vol. 16, No. 5, pp. 439-445, 1998

Printed in Great Britain
0264-410X/98 $19+0.00

Enhancement of MHC class I-restricted peptide-specific T cell induction by a DNA prime/MVA boost vaccination regime

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Human immunodeficiency virus (HIV) vaccine candidates were previously constructed as a string of cytotoxic T lymphocyte (CTL) epitopes delivered and expressed using DNA and modified virus Ankara (MVA; an attenuated vaccinia virus) vectors. These vaccines were shown to induce interferon (IFN)-γ-producing and cytolytic CD8⁺ T cells after a single vaccine administration. In the course of this work, immunization protocols were sought which would improve the levels of induced HIV-specific T cells. It was found that previous immunological exposure to MVA reduced the efficiency of subsequent priming and boosting using the same vaccine vehicle. However, a combined regime whereby the animals were first primed with the DNA vaccine and then boosted with MVA was the most potent protocol for the induction of both interferon-γ-producing and cytolytic T cells against two CTL epitopes simultaneously. The general applicability of this novel vaccination method for induction of major histocompatibility complex class I-restricted T cells is discussed. © 1998 Elsevier Science Ltd. All rights reserved

Keywords: DNA; MVA; vaccine; CTL; polyepitope; HIV

It is recognized that successful vaccines against many infectious diseases have to induce responses mediated by $CD8^+$ T cells. These lymphocytes participate in the organism's defense in more than one way. Firstly, they prevent pathogen reproduction by killing infected cells displaying on their surface 8- to 10-amino acid residue peptides derived from microbial proteins bound to major histocompatibility complex (MHC) class I molecules[1]. Hence they are frequently designated cytotoxic T lymphocytes (CTL). Secondly, $CD8^+$ T cells can secrete a variety of soluble factors such as interferons (IFN)-γ, tumor necrosis factors or chemokines, which directly or indirectly contribute to the control of infections. In particular, it was suggested by gene knockout studies that secretion of IFN-γ is as important as perforin- or Fas-mediated killing for protection against cytopathic viruses[2]. It is also recognized that for practical and/or safety reasons, subunit vaccines rather than vaccines based on whole inactivated or attenuated pathogen preparations are likely to be the choice for some infectious agents such as human immunodeficiency virus (HIV), the etiologic agent of acquired immunodeficiency syndrome (AIDS). Subunit or epitope-based vaccines in general hold much promise for the development of new modern approaches to effectively controlling infectious diseases. Novel HIV vaccine candidates were previously constructed. These were designed as a gene designated H encoding a string of partially overlapping epitopes that are recognized by CTL. This gene was delivered using plasmid pTH DNA[3] or modified vaccinia virus Ankara (MVA) (Hanke *et al.*, submitted) as vaccine vehicles. Direct DNA inoculation is a method for efficient induction of both antibody and CTL responses[3-10]. MVA is a strongly attenuated vaccinia virus strain which grows well in chicken embryo fibroblasts, but virtually lost its ability to replicate in those mammalian cells, that have been tested[11-14]. MVA expresses an appropriate profile of virus-derived cytokine receptors (Blanchard *et al.*, submitted) and was proven to be safe when used in over 120000 humans[15]. The H multi-CTL epitope polypeptide consisted mostly of epitopes recognized by human and macaque CTL, but also contained one epitope recognized by murine CTL. To be able to follow the induction of T cells specific for two different

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epitopes, another polyepitope gene containing one *Plasmodium*-derived murine epitope[16] was coupled to the 3'-end of H gene to generate gene HM[3]. It was demonstrated that a single intramuscular (i.m.) vaccination using 'naked' DNA consistently induced cytolytic and IFN-γ-producing T cells specific for both HIV and *Plasmodium* epitopes[3]. Similarly, a single intravenous (i.v.) or i.m. administrations of MVA induced epitope-specific T cells that lasted for at least 55 days (Hanke *et al.*, submitted). In this study, the possibility of successive prime-boost immunizations to augment the $CD8^+$ T cell induction was explored and one superior vaccination regime was identified.

MATERIALS AND METHODS

Preparation of DNA vaccines and DNA immunization

Plasmid DNA used for immunizations was prepared using the Qiagen Megaprep Columns according to the vendor's protocol. Five- to six-week-old female Balb/c mice were injected into skeletal muscles as described previously[3]. Briefly, fur was removed from both lower hind legs and the calf muscles were injected with 50 μl of 10^{-5} M cardiotoxin, a 60-amino acid-residue peptide isolated from *Naja nigricollis* venom (Latoxan, France) resuspended in 0.9% NaCl to induce muscle regeneration causing an increased DNA uptake. Six days later, total of 100 μg of plasmid DNA in endotoxin-free phosphate-buffered saline (PBS; Sigma) was injected into the same muscles. Fifteen days after the first immunization, mice were reinjected into the same sites with half the amount of cardiotoxin and reimmunized using 100 μg of DNA 6 days later. For MVA boosting, mice were injected into the same sites 21 days after the first vaccination with 10^6 plaque-forming units (p.f.u.) of MVA, i.e. at the same time as the second DNA vaccination. All injections were carried out under general anaesthesia.

Preparation of the MVA vaccine stocks and MVA immunization

Bulk stocks of the recombinant MVA were grown on primary chicken embryo fibroblast (CEF) cells obtained from the eggs of a specific pathogen-free flock. MVA was purified by centrifugation of cytoplasmic extracts through a 36% (w/v) sucrose cushion in a Beckman SW28 rotor at 13500 rev. min^{-1} for 80 min (Hanke *et al.*, submitted). The virus stock titres were determined using CEF cell monolayers. Five- to six-week-old female Balb/c mice were injected into their calf muscles with total of 10^6 p.f.u. of MVA in sterile PBS. Reimmunizations with DNA or MVA were carried out in the same manner as described above for the DNA injection except that the full dose of 50 μl of 10^{-5} M cardiotoxin was used for the DNA boost as this was the first cardiotoxin treatment of these animals. All injections were carried out under general anaesthesia.

CTL cultures

Ten days after the last immunization, spleens were removed and pressed individually through a cell strainer (Falcon) using a 2-ml syringe rubber plunger. The splenocytes were washed twice, suspended in 10 ml of Lymphocyte medium [RPMI 1640 supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin, 20 mM HEPES and 15 mM β-mercaptoethanol] and incubated with 2 μg ml^{-1} of peptide(s) in an humidified incubator in 5% CO_2 at 37°C for 5–6 days. The two peptide epitopes used throughout this study were RGPGRAFVTI derived from HIV-1 and restricted by H-$2D^d$[17], and pb9 epitope SYIPSAEKI derived from *P. berghei* and restricted by H-$2K^d$[18].

Target cells and standard ^{51}Cr-release assay

The effector cells were diluted twofold in U-bottom wells (96-well plate; Costar) to yield 100:1, 50:1 and 25:1 effector to target ratios. Five thousand ^{51}Cr-labeled P815 cells in a medium containing 10^{-7} M peptide was then added to the effectors and incubated at 37°C for 4 h. Spontaneous and total chromium releases were estimated from wells, in which the target cells were kept in a medium alone or 5% Triton X-100, respectively. The percentage specific lysis was calculated as [(sample release − spontaneous release)/(total release − spontaneous release)] × 100. The spontaneous release was lower than 5% of the total c.p.m. For the net percentage release, the no-peptide background percent lysis was subtracted from that of the assay well.

CD4 and CD8 lymphocyte depletion

Ninety six-well ELISA plates were coated with either rat anti-mouse CD4 (Serotech) or rat anti-mouse CD8α (Serotech) mAb at 10 μg ml^{-1} in PBS at 4°C overnight. Next day, wells were washed with PBS and 3×10^6 freshly isolated splenocytes per 9 wells were added, incubated in 5% CO_2 at 37°C for 1 h. The unattached cells were then gently removed and used in the ELISPOT assay.

ELISPOT assay

The enzyme-linked immunospot (ELISPOT) assay for detection of IFN-γ-releasing cells[19] and its modification using specific peptide stimulation[20] were described previously. Nitrocellulose-backed 96-well plates (MAHA S45, Millipore) were coated with 50 μl of 15 μg ml^{-1} of murine IFN-γ-specific mAb R4 (ATCC) overnight at 4°C, washed 6 × with PBS and blocked using a medium supplemented with 10% FBS at room temperature for 1 h. Two dilutions of normal or depleted fresh, or *in vitro* restimulated splenocytes and 2 μg ml^{-1} of a specific peptide were then added into the wells and incubated at 37°C in 5% CO_2 overnight. Splenocytes without peptide were included to estimate the baseline numbers of IFN-γ-producing cells. The cells were washed 3 × with PBS, 1 μg ml^{-1} of secondary biotin-conjugated antibody XMG1.2 (Pharmigen) was added and reacted at room temperature for 3 h. The wells were washed 6 × with PBS and alkaline phosphatase (AP)-labeled streptavidin (Sigma) was added at 1:1000 dilution for 1 h. The wells were washed again 6 × with PBS and the spots were developed by adding AP substrate 3,3'-diaminobenzidine-tetrahydrochloride dihydrate (DAB; Sigma). After

45 min, the wells were washed with tap water, dried and the spots were counted under a dissection microscope.

RESULTS

Correlation between cytolytic and IFN-γ-producing T cells

Recombinant viruses MVA.H and MVA.HM, in which the H and HM designate respective HIV and combined HIV-*Plasmodium* polyepitope genes, were constructed in previous work and shown to induce cytolytic and IFN-γ-producing T cells in mice after a single vaccine administration (Hanke *et al.*, submitted). It was also shown that there was a good correlation between the frequency of peptide-specific cells producing IFN-γ in fresh splenocytes and the cytolytic activity of a 5-day *in vitro* peptide-restimulated cultures.

Here, it was first demonstrated that cells producing IFN-γ detected in the ELISPOT assay are predominantly CD8 positive. Fresh splenocytes were either untreated or panned on anti-CD4 or anti-CD8 plates and incubated with the appropriate peptides. While the frequencies of IFN-γ-producing cells in untreated and CD4-panned cultures were similar, CD8-panning significantly reduced the number of positive signals (*Figure 1*).

To gain more information on the relationship between cytotoxicity and IFN-γ production, peptide ELISPOT and standard ^{51}Cr-release assays were carried out in parallel at days 0 and 6 of the splenocyte cultures. On day 0, a low background frequency of cells producing IFN-γ in the absence of a peptide was observed (*Table 1*). There was a very low (5% specific chromium release for the HIV peptide) killing activity when these splenocytes were added directly to peptide-pulsed target cells. However, 480 or 404 of HIV or *Plasmodium* peptide-specific memory T cells per 10^6 splenocytes produced IFN-γ upon peptide stimulation for 16 h. A 6-day culture of splenocytes, which had been restimulated with both the H-2D^d-restricted HIV and H-2K^d-restricted *Plasmodium* peptides, contained on average 439 of IFN-γ-producing cells per 10^6 cells in the absence of any further stimulation by peptide added into the ELISPOT wells. When peptide was added to the culture for 16 h, the frequencies of HIV and *Plasmodium* peptide-responsive cells increased approximately two to threefold and these same cultures displayed specific lyses of 46% and 27%, respectively, at effector to target ratios of 100:1. The incubation of naive splenocytes with peptides corresponding to MHC class I-restricted CTL epitopes yielded a very low background of IFN-γ releasing cells (data not shown). These data suggest that an activation of memory cells and their expansion to 200–300 effectors per 10^6 of cells (no-peptide ELISPOT frequencies after 6-day restimulation with two peptides) are required for or accompanying the cytolytic activity. This expansion is also reflected in the increase of memory T cells (6-day ELISPOT peptide-induced frequencies). More detailed studies of the peptide-specific populations are underway.

Pre-exposure to MVA decreases subsequent MVA vaccine immunogenicity

Although MVA is an attenuated vaccinia virus strain with a severely restricted replicative ability in most mammalian cells, like other poxviruses, it has a very complex genome with more than 150 open reading frames, most of which will be expressed in MVA-infected cells. Immune responses against these proteins may occlude the induction of the desired responses specific for the pathogen-derived antigens. To address these issues, single and double MVA.HM

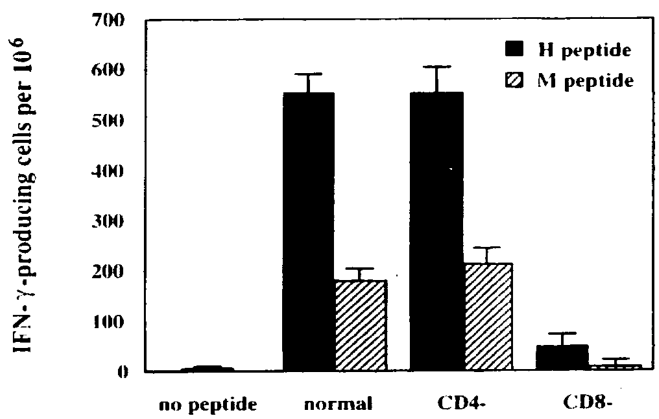

Figure 1 IFN-γ upon peptide stimulation is produced by CD8+ splenocytes. Mice were immunized i.m. with 10^6 p.f.u. of MVA.HM and the frequencies of IFN-γ-producing cells in freshly-isolated untreated, CD4- or CD8-depleted splenocytes were determined in an ELISPOT assay 10 days after the immunization. Full bars and hatched bars indicate HIV and *Plasmodium* peptide specific T cells, respectively. Standard deviations are indicated (n = 2).

Table 1 Correlation of peptide-specific responses determined by ELISPOT and standard ^{51}Cr-release assays of fresh or 6-day *in vitro* peptide-restimulated splenocytes after MVA.HM immunization[a]

	ELISPOT frequencies[b]			% Specific lysis[c]	
Splenocytes	No peptide	HIV	*Plasmodium*	HIV	*Plasmodium*
Fresh	10 ± 7	480 ± 68	404 ± 119	5 ± 2	1 ± 1
6-day peptide-restimulated	439 ± 236[d]	1019 ± 256	606 ± 201	46 ± 2	27 · 6

[a]A group of four mice was immunized with one dose of 10^6 p.f.u. of MVA.HM i.m. and sacrificed 10 days later.
[b]The mean number ± standard deviation of cells producing IFN-γ per 10^6 splenocytes after a 16 h incubation in the absence of (no peptide), or with HIV or *Plasmodium* peptides in the ELISPOT assay wells.
[c]Data shown are the % specific lysis, mean ± standard deviation, at the effector to target ratio of 100:1 in a 4 h ^{51}Cr-release assay. Maximum no-peptide background release was 3%.
[d]This frequency represents the sum of HIV and *Plasmodium* peptide-specific T cells as the splenocytes had been *in vitro* restimulated with both peptides.

vaccinations were compared for their efficiency in inducing cytolytic and IFN-γ-producing T lymphocytes specific for the HIV and *Plasmodium* epitopes. The splenocytes were isolated 10 days after the last immunization and either tested unrestimulated in an ELISPOT assay or after restimulation *in vitro* for 5 days in a standard ^{51}Cr-release CTL assay. It was found that a single MVA dose was on average as efficient as two MVA doses, although the booster improved the consistency of the CTL induction as shown by the tighter error bars (*Figure 2A* and *B*). The ELISPOT frequencies were slightly higher for the double immunization (*Table 2*). Because the single versus double immunization protocol did not question the efficiency of priming in MVA-immune animals, mice were also first immunized with MVA expressing either the HIV polyepitope alone or an irrelevant antigen and then vaccinated with MVA.HM. In the MVA.H/MVA.HM schedule, the CTL responses induced against the HIV epitope were high, while the priming of the *Plasmodium* epitope in the MVA-exposed mice was less efficient compared to both the single and double MVA.HM immunizations (*Figure 2C*). When animals were pre-exposed to an MVA expressing an irrelevant antigen and then vaccinated with MVA.HM, the HIV peptide-specific responses were attenuated relative to the MVA-naive mice in both CTL activities (*Figure 2D*) and ELISPOT frequencies (*Table 2*). Thus, sequential vaccinations using the same complex vaccine vehicle decreased the vaccine immunogenicity both in terms of priming new responses and boosting the existing ones.

DNA prime-MVA boost is the most efficient regime for T cell induction

A DNA vaccine pTH.HM was constructed previously and demonstrated to induce T cell specific for both the HIV and *Plasmodium* epitopes after a single i.m. administration[3]. This vaccine was employed in the next series of immunizations to ascertain whether a higher level of immunogenicity can be achieved using a combination of MVA and DNA vectors expressing the same protein. Firstly, a comparison between single and double DNA vaccinations showed that using the DNA vaccine alone, the CTL responses are boostable (*Figure 3A* and *B*), although this was not reflected in the ELISPOT frequencies of IFN-γ-producing cells (*Table 3*). The overall cytotoxic activity was somewhat lower in this series of immunizations than that observed previously[3] possibly due to animal variation. Finally, combined DNA/MVA vaccinations were carried out. It was established that the most potent regime for inducing T cells was to prime with the pTH.HM vaccine and boost with MVA.HM. This was best seen on the cytolytic activity elicited against the less efficient *Plasmodium* epitope (*Figure 3D*) and the highest average frequencies of 1000 and 748 of the respective HIV and *Plasmodium* peptide-responsive cells per 10^6 of freshly isolated splenocytes (*Table 3*).

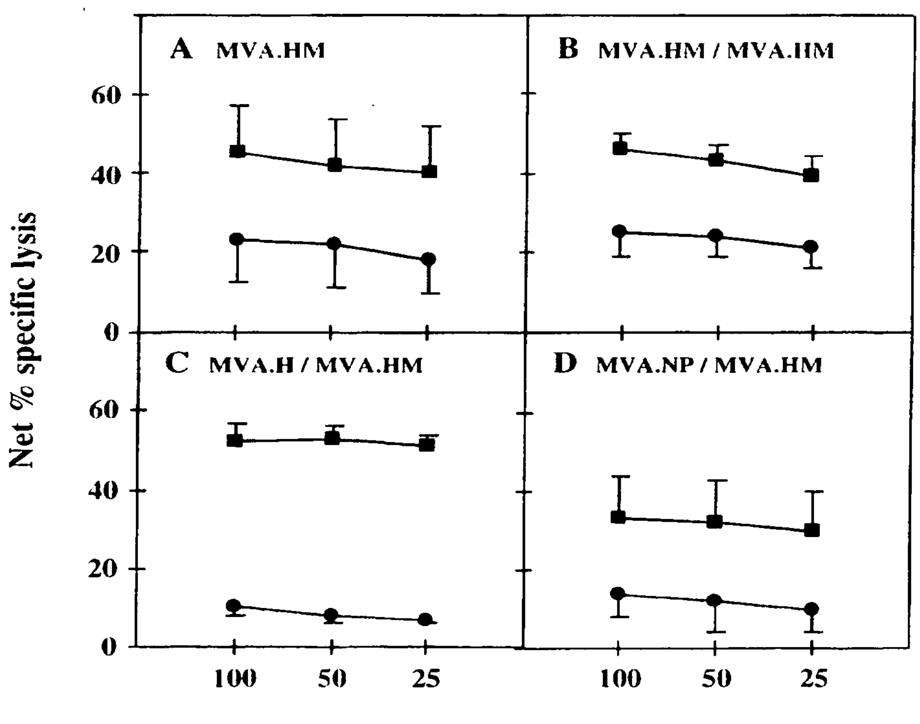

Figure 2 CTL activities elicited by sequential MVA immunizations. Groups of Balb/c mice were immunized once or twice in a 3-week interval using 10^6 p.f.u. of recombinant MVA vaccines. Mice were sacrificed 10 days after the last immunization, the splenocytes from individual mice were isolated and separately restimulated with a mixture of the HIV and *Plasmodium* peptides for 5 days. The elicited cytolytic activities against the HIV (squares). *Plasmodium* (circles) and peptides were determined in an *in vitro* standard 4 h ^{51}Cr-release assay using peptide-pulsed P815 cells as targets. Each point represents an average percentage of net killing ± standard deviation (n = 4) at the indicated effector to target ratios.

DISCUSSION

An ELISPOT assay using peptides corresponding to defined MHC class I-restricted CTL epitopes is a simple, quick, sensitive and non-radioactive method for enumerating epitope-specific release of IFN-γ by individual $CD8^+$ T cells in preparations of lymphocytes[20]. *Table 1* shows that 10 days following i.m. immunization, quiescent memory cells are present in the central lymphoid organ which can be activated to produce IFN-γ by addition of specific peptides. The frequency of cytolytic effectors is too low to detect lysis without an *in vitro* restimulation. The ELISPOT-determined frequencies of IFN-γ-producing cells in fresh unstimulated splenocyte cultures correlated here and in previous studies (ref. 3 and Hanke *et al.*, submitted) with the levels of cytotoxic activity observed after a 5-day *in vitro* peptide restimulation. It is relevant that under similar conditions 50% of cells of a $CD8^+$ cytolytic T cell clone were detected to produce IFN-γ (Lalvani *et al.*, unpublished observations). Because the detection of IFN-γ-producing cells is more sensitive and quantitative than ^{51}Cr-release cytotoxicity assay and the IFN-γ secretion might be as relevant to protection as cytolytic activity, the ELISPOT assay represents a useful method for evaluating the success of vaccinations and complements the ^{51}Cr-release assay.

Table 2 Induction of peptide-specific INF-γ-producing splenocytes by sequential immunizations using MVA-based vaccines

Vaccination[a]		*In vitro* stimulation[b,c]	
Week 0	Week 3	HIV peptide	*Plasmodium* peptide
None	MVA.HM	422 ± 128	212 ± 94
MVA.HM	MVA.HM	786 ± 334	238 ± 106
MVA.H	MVA.HM	546 ± 190	102 ± 86
MVA.NP[d]	MVA.HM	56 ± 52	64 ± 14

[a]A total dose of 10^6 p.f.u. of recombinant MVA.H or MVA.HM was administered into calf muscles under anesthesia.
[b]Splenocytes were stimulated with 2 μg ml^{-1} of peptides in the assay well for 16 h.
[c]The ELISPOT frequencies of IFN-γ-producing cells are shown as a mean number ± standard deviation (n = 4) of responding cells per 10^6 of freshly isolated splenocytes.
[d]MVA.NP expressing the influenza virus nucleoprotein (NP) was used as an irrelevant MVA.

Novel candidate HIV vaccines were designed as an array of human CTL epitopes delivered by a means of plasmid DNA[3] or MVA (Hanke *et al.*, submitted) vectors. Both of the vaccine vehicles were chosen for their acceptability for and potential safety in humans[15]. Murine epitopes were included so that using the same vaccines, regimes for immunization can be optimized for an efficient $CD8^+$ T cell induction in a small animal model prior to primate vaccinations. However, increasing of the DNA uptake by pre-treating the myocytes with cardiotoxin will not be suitable for primate or human vaccination protocols.

In the course of this work, it was found that previous exposure to MVA abates the immunogenicity of MVA-based vaccines (*Figure 2* and *Table 2*). Therefore using MVA, the priming of immune responses in animals previously exposed to or vaccinated with MVA is less efficient and the benefit of subsequent MVA boosters decreases. Taking the advantage of having the

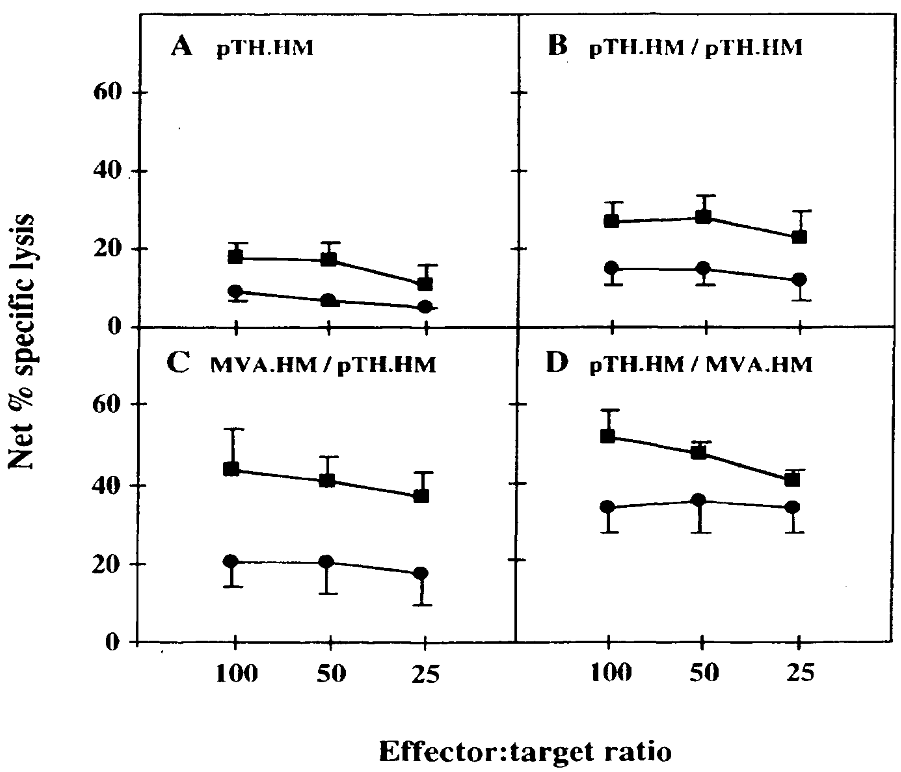

Figure 3 CTL activities elicited by a DNA vaccine alone or in a combination with MVA. Mice were immunized either with 100 μg of pTH.HM DNA or 10^6 p.f.u. of MVA.HM. The immune splenocytes were treated and the induced CTL activities were determined as described in Materials and Methods. Each point represents an average percentage of net killing ± standard deviation (n = 4) at the indicated effector to target ratios specific for the HIV (squares) and *Plasmodium* (circles) peptides.

Table 3 Induction of peptide-specific INF-γ-producing splenocytes using DNA alone or in a combined regime with MVA

Vaccination[a]		*In vitro* stimulation[b]	
Week 0	Week 3	HIV peptide	Plasmodium peptide
None	pTH.HM	70 ± 60[c]	100 ± 10
pTH.HM	pTH.HM	56 ± 26	4 ± 4
MVA.HM	pTH.HM	306 ± 78	58 ± 18
pTH.HM	MVA.HM	1,000 ± 487[d]	748 ± 444[e]

[a]Total doses of 100 μg of pTH.HM DNA were injected into cardiotoxin-pretreated calf muscles or 10^6 p.f.u. of MVA.HM were injected into untreated muscles. All immunizations were performed under general anesthesia.
[b]Splenocytes were stimulated with 1 μg ml^{-1} of peptides in the assay well overnight.
[c]The ELISPOT frequencies of IFN-γ-producing cells are shown as a mean number ± standard deviation (n = 4) of responding cells per 10^6 of freshly isolated splenocytes.
[d]The P values were 0.044, 0.044 and 0.088 for the pTH, pTH/pTH and MVA/pTH immunizations, respectively, relative to the pTH/MVA regime.
[e]The P values were 0.085, 0.062 and 0.074 for the pTH, pTH/pTH and MVA/pTH immunizations, respectively, relative to the pTH/MVA regime.

same antigen in two different vaccine vehicles, combined vaccination regimes were employed and one was identified which induced high levels of CD8$^+$ T cells (*Figure 2* and *Table 3*). In this protocol, the animals were primed with DNA and boosted with MVA. Although only a modest statistical significance was achieved, there was a clear trend in the enhancement of the induced CD8+ T cell responses. Indeed, it was previously shown that the DNA/MVA regime and, in particular, the immunization with the HM polyepitope, resulted in an unexpectedly high levels of protective efficacy (100%) against *P. berghei* challenge (Schneider *et al.*, submitted). To our knowledge, this sequence of immunizations is novel. There is published work involving bimodal immunizations of mice and monkeys, which employed DNA priming and boosting with recombinant vaccinia viruses. However, these data either concerned antibody rather than CTL induction[21,22] or the authors did not observe any benefit in using this particular sequence (Fuller *et al.*, in press), possibly because Western Reserve rather than MVA strain of vaccinia virus was used. We believe that the unique set of MVA-expressed cytokine receptors might be responsible for the difference. Augmentation of DNA elicited immune responses was also shown for boosts with purified protein subunits[22–24].

Finally, a simultaneous increase in the induction of T cells specific for two CTL epitopes derived from two different pathogens is reported. It is reasonable to assume that this regime will be also effective for other CTL epitopes derived from other infectious agents or indeed tumor antigens and that it may be adopted as a general vaccine strategy for induction of high levels of specific CD8$^+$ T cells.

ACKNOWLEDGEMENTS

Grant support from MRC U.K. (A.McM., T.H., G.L.S., T.J.B., M.B.), European Community (J.S.) and The Wellcome Trust (A.V.S.H., S.C.G.) are fully acknowledged.

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